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ENZYMIC OXIDATION OF ALIPHATIC DIAMINES

BY

H. BLASCHKO AND JOYCE HAWKINS

From the Department of Pharmacology, University of Oxford

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Two enzymes are known which catalyse the oxidative deamination of aliphatic amines. One of these was discovered by Hare (1928) in a study of tyramine metabolism; this enzyme is now usually called amine oxidase. The other enzyme is histaminase (Best and McHenry, 1930).

Until 1945 the first of these enzymes was also sometimes called " monoamine oxidase," as it was thought that it acted only on monoamines, whereas the term " diamine oxidase " was introduced for histaminase when it was found that this enzyme acted on diamines like putrescine and cadaverine (Zeller, 1938); this has since been confirmed on more purified preparations (Kapeller-Adler, 1949).

In 1945 it was shown that three straight-chain diamines, $H_2N(CH_2)_{14}NH_2$, $H_2N(CH_2)_{16}NH_2$, and $H_2N(CH_2)_{18}NH_2$, were not oxidized by " diamine oxidase," but that they were substrates of amine oxidase (Blaschko and Duthie, 1945). This observation suggested that the affinity of diamines for the two enzymes was determined by the length of the polymethylene chain separating the two amino groups. A more systematic study of the enzymic oxidation of the diamines of the series $H_2N(CH_2)_nNH_2$ has therefore been made, and the results of this study are described in this paper.

MATERIAL AND METHODS

Enzymic preparations.—The work on amine oxidase has been greatly helped by an observation made with Mrs. I. Wajda in 1945 that the enzymic activity of the tissues is not impaired by treatment with ice-cold acetone. Since that time, most of our experiments have been carried out with acetone-dried powders. One of the advantages of the method is that it is easy to obtain batches of material with known and stable enzymic activities. Moreover, the total enzymic activity resides in the insoluble particles; the preparations can therefore be washed repeatedly with 0.067 M-sodium phosphate buffer and centrifuged. The washings do not have any enzymic activity. This treatment considerably reduces the oxygen consumption of the enzyme blank. In the present experiments, rabbit liver was used. The powder was suspended in phosphate buffer and centrifuged. The supernatant was discarded and fresh phosphate buffer added. This procedure was repeated twice. The preparation of amine oxidase from pig's kidney used in a few experiments will be described in the experimental part of the paper.

The preparation of histaminase from pig's kidney did not essentially differ from that employed by Blaschko and Duthie (1945), except that a Waring blender was used to produce a more thorough and rapid mixing of tissue and acetone.

Substrates.—Hexamethylenediamine dihydrochloride was recrystallized from a commercial specimen. All the other diamines, also as dihydrochlorides, were kindly given to us by Dr. H. King, F.R.S., and Dr. E. J. Zaimis.

Manometric procedure.—All experiments were carried out in conical Warburg flasks with one side bulb and an inner potash cylinder; the latter carried a filter paper and 0.3 ml. N-KOH. The gas phase was oxygen and the temperature of the bath 37°.

Observations on amine oxidase

Chain length and oxidation rate

Amine oxidase of rabbit liver.—In these experiments a suspension of either 40 or 80 mg. rabbit liver powder in 1.6 ml. sodium phosphate buffer was present in each flask. The side arms contained 0.4 ml. of a 0.05 M-diamine dihydrochloride solution so that the initial substrate concentration was 0.01 M. Readings were taken at 5-minute intervals for half an hour, and the 0–15 minute readings were used for comparing the rates of oxygen uptake with the different substrates. In each experiment one flask contained the diamine with eleven carbon atoms, undecamethylene diamine. This substance served as a basis of comparison.

TABLE I

OXIDATION OF DIAMINES $H_2N(CH_2)_nNH_2$ BY AN ACETONE-DRIED PREPARATION OF RABBIT LIVER (AMINE OXIDASE)

Exp.	Oxygen uptake as per cent of that with C_{11} diamine									
	C_6	C_7	C_8	C_9	C_{10}	C_{11}†	C_{12}	C_{13}	C_{14}	C_{15}
1	—	—	—	25	60	100 (10)	105	175	—	—
2	—	—	—	41	65	100 (8)	94	112	—	—
3	—	—	—	—	—	100 (10)	160	150	175	—
4	—	—	—	—	—	100 (13)	111	193	93	—
5	—	—	—	—	—	100 (10)	—	195	165	—
6	—	—	—	—	—	100 (16)	—	145	122	—
7*	9	39	44	39	—	100 (9)	—	—	—	—
8*	4	18	27	32	—	100 (11)	—	—	—	—
9	—	—	—	—	—	100 (33)	159	142	154	121
10	—	—	—	—	—	100 (30)	160	160	165	145
11*	2	53	42	—	—	100 (18)	—	—	—	—
Average ..	5	37	38	34	62	100 (15)	131	153	146	133

* The experiments marked with an asterisk were carried out with 80 mg. of the liver powder in each flask; in all the other experiments the amount of liver powder was 40 mg.
† The figures in parentheses are μl. O_2 consumed in the first 15 minutes.

Table I gives the results of all the experiments. In each single experiment the amount of oxygen taken up in the flask with the C_{11} compound is given as 100; the figures given in parentheses are the μl. of O_2 consumed by 40 mg. of powder in 15 minutes. The oxygen consumptions with the other diamines are given as per cent of those with undecamethylenediamine.

The Table shows a close relation between the rate of oxidation and the number of methylene groups in the chain. The rate of oxidation increased with increasing

chain length up to the compound with thirteen carbon atoms; on increasing the chain length even further the rate of oxidation declined. The C_6 compound was not significantly oxidized; the figures for oxygen uptake with the C_7, C_8, and C_9 compounds are small, but outside the limits of experimental error.

Amine oxidase of pig kidney.—In a later section of this paper our experiments with preparations of histaminase from pig's kidney will be reported. It seemed of interest to find out if the lower end of the specificity range of the amine oxidase in pig's kidney was similar to that found for the amine oxidase of rabbit's liver.

In these experiments it was essential to find a preparation in which histaminase activity did not contribute to the oxygen consumption. The following procedure was adopted: 100 ml. of phosphate buffer were added to 0.5 g. of the acetone-dried powder of pig's kidney; the suspension was frequently stirred and eventually the supernatant fluid was decanted and discarded. This treatment of the sediment with phosphate buffer was repeated four times; the last time the suspension was allowed to stand overnight at +2°. The sediment was then suspended in 9 ml. phosphate buffer. This final suspension was used in the experiment. The manometer flasks were filled as follows: the main compartment contained 1.4 ml. of the suspension and 0.2 ml. 0.1 M-semicarbazide; the side arm contained 0.4 ml. water in the flask which served as enzyme blank, and 0.4 ml. 0.5 M-diamine dihydrochloride in the other flasks.

This procedure was adopted because most of the histaminase is eluted from the sediment by the phosphate buffer and any remaining histaminase activity is inhibited by semicarbazide.

The diamines tested with the pig kidney preparation were those with 6, 7, 8, and 11 CH_2-groups. Table II, which gives the results of two such experiments, shows that the C_7, C_8, and C_{11} diamines were oxidized. The relative rates of oxidation were similar to those found with rabbit liver. The absence of any significant oxygen uptake with the C_6 diamine proves that histaminase did not contribute to the oxidation of the other diamines.

TABLE II

OXIDATION OF DIAMINES BY WASHED SUSPENSIONS OF PIG'S KIDNEY POWDER

Exp.	Oxygen uptake as per cent of that with C_{11} diamine			
	C_6	C_7	C_8	C_{11}*
1	6.5	35	69	100 (16)
2	7.0	28	31	100 (14.5)

* The figures in parentheses are O_2 uptake in μl. O_2/15 minutes.

Competition of diamines for amine oxidase

The experiments on substrate specificity for amine oxidase were supplemented by observations which suggest that parallel to the increase in oxidation rate with increasing chain length there occurs an increase in the affinity for the enzyme. This is illustrated by the two experiments shown in Fig. 1. We have chosen nona-

methylene diamine as a representative of a relatively poor substrate and tridecamethylene diamine which shows the highest rate of oxidation in this series.

In these experiments each flask contained a suspension of 30 mg. of rabbit liver powder in 1.4 ml. sodium phosphate buffer plus 0.2 ml. 0.1 M-semicarbazide. The semicarbazide was added in order to exclude oxygen uptake due to the further

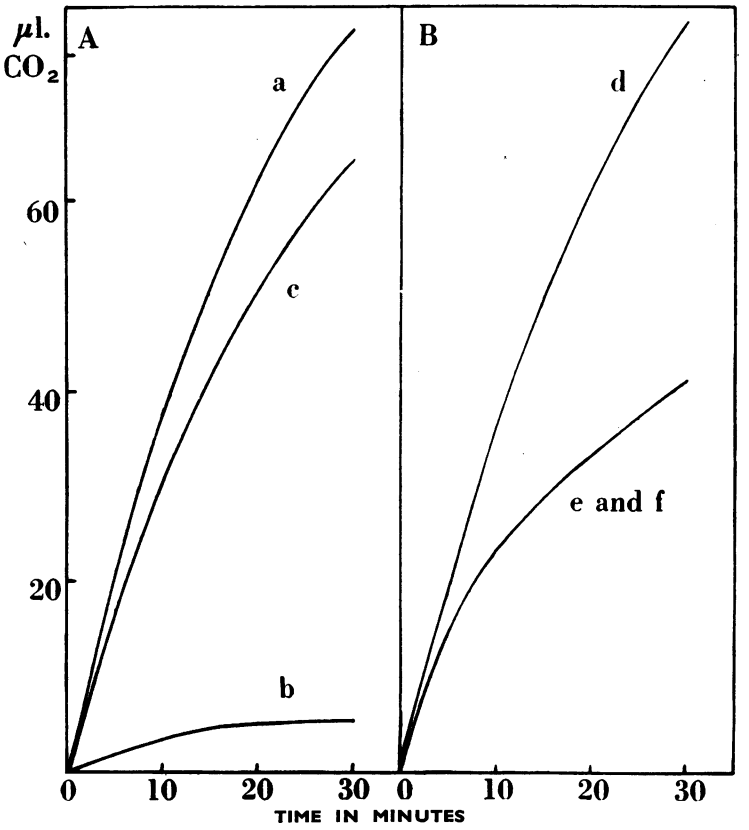

FIG. 1.—Rabbit liver powder (amine oxidase). Mixed substrate experiments. (A) Curve *a*: oxygen uptake with 0.01 M-*iso*amylamine; curve *b*: oxygen uptake with 0.01 M-nonamethylene diamine; curve *c*: oxygen uptake with both amines present. (B) Curve *d*: oxygen uptake with 0.01 M-*iso*amylamine; curve *e*: oxygen uptake with 0.01 M-tridecamethylene diamine; curve *f*: oxygen uptake with both amines present.

oxidation of the aldehyde formed in the enzymic reaction (Bhagvat, Blaschko, and Richter, 1939). The side bulbs contained *iso*amylamine and/or the diamine to be investigated. The initial concentration of the amines after tipping was 0.01 M. In Fig. 1 the oxygen uptake is plotted against time. In the experiment of Fig. 1A the rate of oxygen uptake in the presence of both *iso*amylamine and the C_9 diamine was about intermediate between the rate with either substance alone. With *iso*amylamine and the C_{13} diamine, however (Fig. 1B), the rate of oxygen uptake was practically determined by the C_{13} diamine. This indicates that tridecamethylene diamine had a much higher affinity for amine oxidase than *iso*amylamine.

OBSERVATIONS ON HISTAMINASE

Diamines as substrates of histaminase

In these experiments each flask contained 1.6 ml. of the extract of pig kidney powder equivalent to 160 mg. of powder; the side arm carried 0.2 ml. 0.05 M-diamine dihydrochloride and 0.2 ml. water. Readings were taken every five minutes for 30 minutes; the experimental figures given are based on the oxygen uptake during the first 15 minutes. Hexamethylene diamine was used as a standard of comparison in these experiments. In Table III results are given as per cent of the rate of oxidation of hexamethylene diamine. The average amount of oxygen consumed during 15 minutes with this substance was 20 μl. O_2. With the extracts of pig's kidney the blank oxygen consumption is greater, and a reading of 2 μl. O_2 consumed—that is, 10 per cent of the rate of oxidation of hexamethylene diamine—is scarcely outside the limits of experimental error.

TABLE III

OXIDATION OF DIAMINES $H_2N(CH_2)_nNH_2$ BY AN EXTRACT OF PIG KIDNEY (HISTAMINASE)

Exp.	Oxygen uptake as per cent of that with hexamethylene diamine										
	C_4	C_5	C_6*	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}
1 ..	162	176	100 (21)	103	31	—	—	—	—	—	—
2 ..	175	172	100 (18)	100	53	—	—	—	—	—	—
3 ..	—	—	100 (20)	—	33	25	15	15	—	—	—
4 ..	—	—	100 (18.5)	—	13	0	24	32	—	—	—
5 ..	—	—	100 (20)	—	—	12	—	—	10	12	10
6 ..	—	134	100 (29)	90	8	—	—	—	—	—	—
7 ..	—	127	100 (25.5)	98	37	—	—	—	—	—	—
8 ..	—	—	100 (27.5)	—	—	16	—	—	—	—	—
9 ..	—	—	100 (9)	—	—	—	11	0	—	11	—
10 ..	—	—	100 (20.5)	—	—	—	5	0	0	—	—
11 ..	—	—	100 (19.5)	—	—	—	0	2	0	—	—
Average	168	152	100 (21)	98	29	13	11	10	3	12	10

* The figures in parentheses are μl. O_2 consumed in the first 15 minutes.

In the series of substances examined, the rate of oxidation was highest with putrescine; the rate for the higher members of this series was progressively less; with the diamines with 10 to 14 CH_2-groups it was probably within the limits of experimental error.

Competition experiments

It has already been shown (Blaschko and Duthie, 1945) that the long-chain diamines with 14 and 16 CH_2-groups, although not oxidized by histaminase, are not without affinity for the enzyme; they act as inhibitors. The same is true for the lower members of the series; we have made one experiment in which the influence

of the C_9 and the C_{13} diamines on the rate of oxidation with cadaverine was studied. The oxygen uptake in the 5–35 minute interval was:

with cadaverine alone	78.5 µl.
,, nonamethylene diamine alone	4.5 µl.
,, tridecamethylene diamine alone	1.5 µl.
,, nonamethylene diamine plus cadaverine	55 µl.
,, tridecamethylene diamine plus cadaverine	45 µl.

The oxygen consumption in the enzyme blank was 13.5 µl. O_2 in the same period : it has been subtracted.

Discussion

The observations reported in this paper show clearly that in the homologous series $H_2N(CH_2)_nNH_2$ substrate specificity is determined by the length of the polymethylene chain which separates the two amino groups. Histaminase acts on

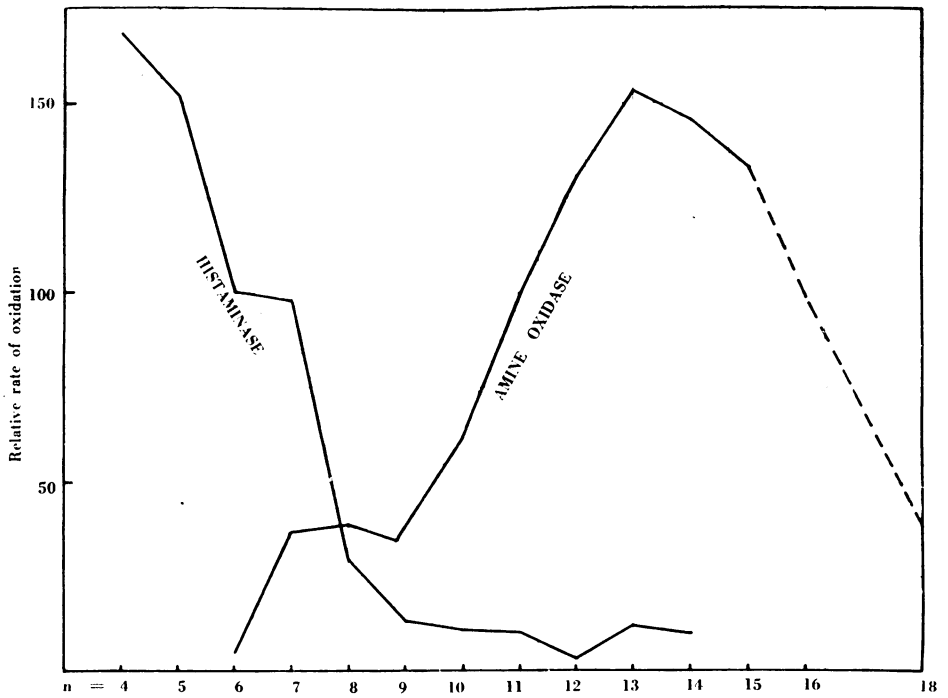

FIG. 2.—Oxidation of the polymethylene diamines $H_2N(CH_2)_nNH_2$ by histaminase and by amine oxidase. Abscissae: number n of CH_2-groups; ordinates: relative rate of oxidation in arbitrary units (see text). The results obtained by Blaschko and Duthie (1945) with the C_{16} and C_{18} diamines are represented by the dotted line.

the short-chain compounds. Amine oxidase acts on the longer members of the series, the optimum chain length being at $n = 13$. The result of this study of specificity is summarized in Fig. 2, in which the rates of oxidation with the two enzymes are plotted against chain length. The results with the C_{16} and C_{18} diamines

obtained with a different preparation of amine oxidase from rabbit liver (Blaschko and Duthie, 1945) have been included.

Whereas it is not difficult to determine the optimum configuration for each enzyme, it is less easy to be certain about the range of specificity. However, it seems certain from our results that the C_7 and C_8 diamines are poor substrates of both enzymes. In fact, in the pig's kidney there occur two enzymes which will oxidize these two compounds.

We know now that the mammalian organism contains catalysts which enable it to dispose of all the diamines examined, up to a chain length of eighteen carbon atoms by oxidative deamination. On the other hand, there seems to exist no enzymic mechanism able to act on the corresponding bis-quaternary bases. For instance, we have not found any oxygen uptake with the synthetic curare substitute decamethonium bisiodide (Barlow and Ing, 1948; Paton and Zaimis, 1949); in agreement with this Zaimis observed (1950) that hexamethonium appears unchanged in the urine.

Since the first observation on the enzymic oxidation of diamines by amine oxidase other diamines have been reported to be substrates of this enzyme. These belong to the series of aromatic alkylene α: β-diamines studied by Lehmann and Randall (1948), especially the two compounds Nu 1408 and Nu 1683. It is interesting that these two diamines are also pressor amines. MacIntosh and Paton (1949) state that the C_{16} diamine " appears to combine the vasoactive properties of long-chain primary monoamines (Barger and Dale, 1910) with those of the shorter diamines." Thus, in our series as well as in that examined by Lehmann and Randall (1948) there are pressor agents which have an affinity for amine oxidase. The parallelism between affinity for amine oxidase and pressor response, however, does not appear to be strict: MacIntosh and Paton did not report any pressor action for dodecamethylene diamine, which is a good substrate of amine oxidase.

That the size of the alkyl group is important for the oxidation of aliphatic amines by amine oxidase has long been known (Pugh and Quastel, 1937; Blaschko, Richter, and Schlossmann, 1937). Alles and Heegaard (1943) reported that the rabbit liver enzyme did not act upon methylamine, ethylamine, and propylamine, but that butylamine and the higher homologues were oxidized. Dodecylamine is the highest member of this series known to be a substrate (Blaschko and Duthie, 1945). The introduction of the second basic group, as in diamines like putrescine or cadaverine, abolishes substrate specificity. The experiments of this paper show that the adverse effect of the second basic group on specificity is progressively lessened with increasing length of the polymethylene chain, and that the long-chain diamines are able to attach themselves to amine oxidase like the monoamines. The decrease of the rate of oxidation with the longest members of our series may well be due to their lower solubility, but quantitative data on the solubility of long-chain diamines appear not to be available.

For histaminase (" diamine oxidase ") Zeller (1942) has reported that the maximum rate of oxidation occurs with the C_4 compound, putrescine. He assumes that the substrate molecule is anchored to the enzyme with the two basic groups. We must therefore conclude that enzyme and substrate form an intermediate ring compound and that this ring compound forms less and less readily as the number of carbon atoms separating the two basic groups increases beyond four.

The relationship between pressor action and affinity for amine oxidase finds its parallel in that between the affinity for histaminase and depressor action. Again, we note that the parallelism is imperfect. Cadaverine (pentamethylene diamine) has a depressor effect (Barger and Dale, 1910; MacIntosh and Paton, 1949), but the C_4 diamine, putrescine, is without this effect although it is an even better substrate of histaminase. We conclude that in each instance the enzyme protein has properties somewhat similar to, but not identical with, the protein of the excitable structures in the tissues.

Another question is raised by the experiments here described: Does the affinity of the diamines for histaminase play a part in their action on the blood histaminase level described by MacIntosh and Paton (1949)? This question can at present not be answered; it is not known to what extent the blood histaminase level is normally determined by the action of histaminase. Some of the substances examined by MacIntosh and Paton have little affinity for histaminase, but others have a strong inhibitory action (Blaschko and Duthie, 1944; Blaschko, Fastier, and Wajda).

SUMMARY

1. The oxidation of the polymethylene diamines $H_2N(CH_2)_nNH_2$ (n from 4 to 15) by amine oxidase and histaminase has been studied.

2. Amine oxidase (an acetone-dried preparation) from rabbit liver does not act on the lower members of this series, but the higher homologues were oxidized with a maximum rate at $n = 13$; with longer chains the rate of oxidation decreased.

3. Amine oxidase from pig kidney had a pattern of substrate specificity similar to that of rabbit liver.

4. Competition experiments are reported which show that the C_{13} diamine has a much higher affinity for amine oxidase than the more slowly oxidized C_9 diamine.

5. Histaminase ("diamine oxidase") from pig kidney oxidized putrescine ($n = 4$) most rapidly; with increasing length of the polymethylene chain the rate of oxidation decreased, until for the diamines with 9 or more carbon atoms oxygen uptake was practically nil.

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